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Simultaneous determination of allantoin and glycolic acid in snail mucus and cosmetic creams with high performance liquid chromatography and ultraviolet detection

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ABSTRACT

A new methodology for simultaneous quantitative analysis of allantoin and glycolic acid in snail mucus and cosmetic creams was developed. HPLC separation was achieved a Synergi-Hydro RP column within 7 min using isocratic elution with potassium phosphate (pH 2.7; 10 mM) at a flow rate of 0.7 mL/min at 30 °C. Sample pretreatment was performed by dilution of mucus or cosmetic cream in the elution buffer, heating at 60 °C for 20 min, adjusting the pH to 2.9 and purification with hexane extraction. Linearity was determined with spiked samples and the LLOQ values of 0.0125 and 0.2500 mg/mL were determined for allantoin and glycolic acid, respectively. Accuracy and intra- and inter-day repeatability were studied at three levels of concentrations (0.04, 0.08 and 0.16 mg/mL for allantoin and 0.1, 1.5 and 4.0 mg/mL for glycolic acid) using spiked mucus and cream base samples; mean values of recovery were in the range of 96.81–102.42% in all matrices tested, whereas the respective RSDs (%Relative Standard Deviation) were less than 3.04% in all cases. Spiked mucus and cream samples were stable (RSD < 4.16 and relative error < 4.34%) at room temperature and at 4 °C for 1 week and at −18 °C for 6 months; samples were also stable after three freeze-thaw cycles. The method was applied to the analysis of different lots of snail mucus, and of three commercial creams containing snail mucus.

1. Introduction

Allantoin, the final catabolic product of purines in mammals, has long been used in cosmetics and in medicine without having shown toxicity or undesired side-effects [1,2]. According to USA Food and Drug Administration (FDA), allantoin is a safe and effective active compound for skin protection [3] at a dosage range 0.5–2.0% [4]. Glycolic acid is the most widely used alpha-hydroxy acid for skin care cosmetic products and for the treatment of skin diseases including actinic keratosis, hyperkeratosis and seborrheic eczema [5]. Glycolic acid and its common salts and esters are safe for use in cosmetic products at concentrations ≤10%, and at final pH ≥ 3.5 [6].

Snail mucus has been used in medicine from ancient times for pain relief, the treatment of burn injuries, other injuries and various diseases [7]. In recent years, research on the secretions of the snail *Helix aspersa* have confirmed that the mucus contains an unusual combination of natural ingredients with beneficial and therapeutic qualities for human skin, including allantoin and glycolic acid [8,9].

Many analytical methods have been developed for the determination of allantoin. The commonest spectrophotometric determination is based on the Rimini–Schryver reaction [10]. Allantoin is also estimated in cosmetic and pharmaceutical products with alcaline titration [11,12], and infrared spectrometry (IR) [1]. Determination of allantoin with HPLC in biologic samples is combined with the analysis of other purine products like xanthines, hypoxanthines and uric acid [13–18]. Application of reversed phase HPLC for the estimation of allantoin as ingredient in pharmaceutical and cosmetic products, has also been reported [19,20]. Allantoin is loosely held on a C_{18} reverse phase column; thus, for a good separation and sensitivity either a longer column (30 or 60 cm) or a modified column securing a larger retention time is required [1].

Chromatographic determination of the small chain carboxylic acids such as glycolic acid, is based on gas chromatography after derivatization [21–23] and liquid chromatography [24–29]. Different liquid chromatographic separation modes have been used, i.e. ion exclusion [24,27], ion-exchange [26], reversed-phase [29,31] and ion-pair chromatography [28,30]. Selective and fast estimation of glycolic acid in cosmetic products was suggested using the technique of ion-pair RP-HPLC [30]. Strong-anion exchange

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purification enabled direct reversed phase HPLC-UV determination in cosmetics without prior derivatization [32].

To the best of our knowledge there are no reports of simultaneous allantoin and glycolic acid determination in cosmetic products and raw material; the determination of those compounds in cosmetic products has been performed separately and using different HPLC methods. In this study, a fast and simple HPLC methodology for simultaneous determination of allantoin and glycolic acid in snail mucus and cosmetic creams was developed and validated.

2. Experimental

2.1. Reagents and chemicals

Snail (*H. aspersa*) mucus (secretions) was kindly provided by Helix ir (Crete, Greece). Three cosmetic creams (face creams) with snail mucus as main ingredient were obtained from: (a) Helix ir (Crete, Greece); (b) Lacofar Y Cia, LTDA, (Chile) with brand name Elicina®; and (c) Hudson Laboratory S.A. (Chile) with brand name Labcconte®. The Elicina® and Labcconte® creams were purchased from a local pharmacy store, while the Helix ir cream and the respective cream base were kindly provided by the producer. High quality standard allantoin was supplied from FLUKA (St. Louis, USA) with purity >98.0% and glycolic acid from Sigma–Aldrich (St. Louis, USA) with purity of 99.0%. The reversed phase Synergi 4U Hydro-RP 80A (250 mm × 4.60 mm, 4 μm) column, was acquired from the Phenomenex® (Torrance, CA, USA).

HPLC-grade acetonitrile (CH_3CN) and methanol (CH_3OH) were purchased from Honeywell Burdick & Jackson (Seelze, Germany), mono-potassium phosphate (KH_2PO_4), phosphoric acid (H_3PO_4) and potassium hydroxide (KOH) from Merck KgaA (Darmstadt, Germany), and *n*-hexane ($CH_3(CH_2)_4CH_3$) from Fisher Scientific (Hampton, UK). Ultra-pure water from a MilliQ® instrument (Millipore, Billerica, USA) was used. All solvents filtered through 0.22 μm filters (Titan Membrane, Millipore).

2.2. Sample preparation

Samples spiked with allantoin and glycolic acid were prepared for linearity experiments in either 25 mL of mucus sample solution (1250 μL mucus diluted in elution buffer), or in 50 mL cream base solution (200 mg cream base dissolved in elution buffer). Samples spiked with allantoin at concentrations of 0.005, 0.0125, 0.0250, 0.0500, 0.1000, 0.1500, 0.2000 mg/mL and glycolic acid at 0.05, 0.25, 0.50, 1.00, 2.00, 3.00 and 4.50 mg/mL were prepared.

For the rest of validation experiments, spiked samples were prepared at three concentration levels: 0.16, 0.08, 0.04 mg/mL of allantoin and 4.0, 1.5, 0.4 mg/mL of glycolic acid. These concentrations were prepared by weighing the appropriate amounts (4, 2, 1 mg of allantoin and 100, 37.5, 10 mg of glycolic acid, respectively), and adding them in 25 mL of mucus or 50 mL cream base solution before sample treatment.

2.3. Sample treatment

Cream (200 mg) was dissolved in 50 mL potassium phosphate buffer (pH 2.7; 10 mM) with the aid of sonication for 10 min, followed by heating the solution at 60 °C for 20 min. The solution was stirred until room temperature equilibrium, and its pH was regulated at 2.9 using 10 M KOH. In order to get rid of lipophilic ingredients, the solution (2 mL) was purified with extraction with equal volume of hexane twice. The aqueous solution is filtrated through 0.22 μm filters (Titan Membrane, Millipore).

Mucus (1250 μL) is dissolved in 25 mL of the mobile phase and heated in a waterbath at 60 °C for 15 min. After stirring using a Vortex device for about 2 min, the solution was left to cool to room temperature. Further treatment took place as described for cosmetic cream samples, i.e. pH regulation, extraction with hexane and filtration of aqueous phase.

2.4. HPLC determination

The chromatographic system consisted of an Ultimate 3000 Pump (Pump LPG-3400 A, Dionex Corporation Sunnyvale, CA, USA) with a 20 μL Rheodyne 8125 injector (Rheodyne, Ronhert Park, CA, USA). The Column Compartment (TCC-3100) was stabilized at 30 °C and detection was performed with a Diode Array Detector (DAD), Ultimate DAD-3000. Data were collected, stored and integrated on a Chromeleon v 6.80 Systems software. Separation of analytes was performed on a Synergi Hydro-RP C-18 reversed-phase column (250 mm × 4.6 mm I.D., 5 μm particle size) from Phenomenex (Torrance, CA, USA). Elution was performed with potassium dihydrogen phosphate (pH 2.7; 10 mM) for 15 min. For the complete clean-up and conditioning of the column, at the end of the 15 min isocratic elution the acetonitrile percentage was linearly raised to 70% within 10 min and kept at this percentage for 10 min and then was lowered to 0% within 5 min and kept there for 10 min. The flow rate of the mobile phase was 0.7 mL/min. Elution was monitored at 200 nm.

3. Results and discussion

3.1. Method development

3.1.1. Optimization of chromatographic conditions

Silica-based reverse phase columns like RP, C_{18} and C_8 are widely used for the separation of small molecules. However, these columns are not appropriate for the retention and separation of highly polar compounds. Some modified RP columns, like the Synergi Hydro-RP column, have the capability of partially retaining polar compounds. It is also designed for the use of 100% aqueous phases and an operating range of $2.0 \leq pH \leq 8.0$, and, thus, it was selected for the simultaneous determination of glycolic acid and allantoin.

The effect of mobile phase pH was studied in association to the time of retention, the peak height as well as the peak shape. Using 10 mM KH_2PO_4, buffer solutions of different pH values were prepared by adding phosphoric acid (0.1 M). After comparing the curves in the resulting plots of pH/time, the optimal pH of the mobile phase was found to be 2.7. Increasing the concentration of the mobile phase to 20 mM KH_2PO_4 did not affect the peak shape and the retention time of analytes. Three different flow rate values for the mobile phase (0.5, 0.7 and 1.0 mL/min) were tested. A good separation was achieved within 7 min for the flow rates of 0.5 mL/min and 0.7 mL/min. The value of 0.7 mL/min was chosen for all experiments. The effect of temperature was also studied, by analyzing the standard samples at 30, 35 and 40 °C. However, the different temperature values had little effect on compound separation, so the temperature of 30 °C was chosen. Detection of allantoin and glycolic acid was done at 200 nm (maximum absorbance) after spectra monitoring with the DAD detector.

Both compounds are highly polar and interact loosely with a usual reversed phase C_{18} column material, but in the case of the modified Synergi-Hydro RP, they interact with the column polar endcapping (mainly via H-bonding). In these conditions, allantoin (4.24 ± 0.02 min) elutes earlier than glycolic acid (4.58 ± 0.02 min). The low buffer pH (1 unit lower than the glycolic acid pK_a value of 3.83) denotes that glycolic acid is not ionized, whereas allantoin molecule carries a positive charge (pK_a of 8.48).

Table 1
Linearity data (line equation with a standard calibration curve) in spiked samples of snail mucus and cream base Helix ir ($n = 5$ for each concentration level).

Component	Line equation	R^2	LLOD (mg/mL)	LLOQ (mg/mL)
Allantoin	Mucus $Y = 33.69 \times 10^{-2} + 693.48 \times X$	0.9999	0.004	0.0125
	Cream $Y = 25.04 \times 10^{-2} + 699.06 \times X$	0.9996		
Glycolic acid	Mucus $Y = 80.173 \times 10^{-2} + 21.64 \times X$	0.9997	0.130	0.2500
	Cream $Y = 68.895 \times 10^{-2} + 21.63 \times X$	0.9998		

The specificity of the method was tested (Supplementary Information, Fig. S1); allantoin and glycolic acid are well separated from the structurally related compounds urea (elution time 3.93 ± 0.06) and diazolidinyl urea (5.40 ± 0.03 min), which are common cosmetic ingredients.

3.1.2. Development of pre-treatment procedure

Direct dilution of the mucus in water or buffer was not feasible and led to poor recoveries, bad separation and column overloading due to the presence of many impurities. Precipitation with four times volume of organic solvent (ethanol or acetone) and analysis of the supernatant, led to a significant loss of glycolic acid (over 50%) and partial of allantoin; possibly glycolic acid is caged in the precipitate of polysaccharides/proteins.

Dissolving the cosmetic cream/mucus in water or buffer solution with the aid of ultrasonic bath for 10 min, heating at 60 °C for 20 min, and then filtration and direct analysis with HPLC was performed as earlier suggested [32]; good separation was achieved but the recovery levels were at about 80%. These results led us to focus on the investigation of extraction conditions. The pH of the sample was adjusted after heating; the values of 2.6, 2.9, 3.5, 4.0, 4.5, 5.0 and 8.5 were studied and the pH 2.9 was selected as optimum (high recoveries close to 100%). For purification of samples, extraction with hexane was practiced for the removal of hydrophobic ingredients of cosmetic creams.

3.2. Method validation

The qualitative characteristics of typical chromatograms of the snail mucus and of a cosmetic cream have been determined. The system suitability parameters, i.e. theoretical plates (N), asymmetry factor (A), retention factor (K), resolution (Rs), were calculated by the software they were in accordance with the criteria reported in European Pharmacopoeia ($N > 2000$, $A \leq 2$, $Rs > 1.5$, $K > 2$) (Table S1 in Supplementary Information). Allantoin and glycolic acid in snail mucus and cosmetic creams, were quantified using the constant addition method, comparing the peak areas of each analyte in pure samples to the areas of the spiked samples. The validation of the developed analytical method included evaluation of: (1) linearity, (2) precision (repeatability and intermediate precision), (3) accuracy, and (4) stability according to European Medicines Agency guidelines.

3.2.1. Linearity

For the construction of calibration curves with spiked samples the method of constant addition was used. The calibration curves of the corrected peak areas (in mAU × min – the peak areas of blank samples were subtracted from those of the spiked samples) versus concentration (mg/mL) were linear in the range studied. The data from the calibration curve are summarized in Table 1. Lower limit of Detection (LLOD) values were determined as the concentration giving a peak height three times the noise background and Lower Limit of Quantitation (LLOQ) values as the lowest concentrations giving analysis of acceptable precision [Relative Standard Deviation (RSD) < 20%) and accuracy [Relative Error (RE) < 20%].

3.2.2. Precision and accuracy

Accuracy and precision of the method was evaluated using spiked samples at three levels of concentration (Table 2). The mean recoveries for the two compounds, which were near 100% for

Table 2
%RSD and recoveries for three consecutive days in spiked samples of snail mucus and cream base Helix ir ($n = 5$ at each concentration level).

Component	Theoretical concentrations (mg/mL)		Snail mucus		Cream base	
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Allantoin	0.16	Day 1	98.27	2.64	97.09	0.17
		Day 2	97.65	3.08	96.69	0.32
		Day 3	98.10	2.93	97.67	2.37
		Total	98.01	2.88	97.15	0.98
	0.08	Day 1	97.36	2.76	99.08	0.22
		Day 2	96.33	1.87	99.08	0.22
		Day 3	96.73	0.94	98.53	2.23
		Total	96.81	1.86	98.89	0.89
	0.04	Day 1	97.99	1.07	98.64	0.12
		Day 2	99.75	4.80	98.64	0.12
		Day 3	98.15	3.08	103.04	3.98
		Total	98.63	2.98	100.11	1.41
Glycolic acid	4.00	Day 1	98.67	3.49	102.03	3.74
		Day 2	103.10	2.70	98.38	3.74
		Day 3	97.12	1.41	97.38	1.63
		Total	99.63	2.53	99.26	3.04
	1.50	Day 1	101.42	1.77	107.09	3.77
		Day 2	97.84	2.50	97.98	2.21
		Day 3	97.35	3.26	98.51	0.98
		Total	98.87	2.45	101.19	2.32
	0.40	Day 1	98.13	4.63	107.76	3.03
		Day 2	102.82	3.07	99.07	3.63
		Day 3	100.70	1.30	100.4	2.41
		Total	100.55	3.00	102.42	3.02

Table 3
Values of allantoin and glycolic acid in three different lots of snail mucus (concentration in mg/L) and three different cosmetic creams (concentration mg/g) ($n = 3$).

Products	Allantoin	Glycolic acid
Snail mucus (mg/L) lot 1	48.61 ± 0.60	3753.01 ± 135
Snail mucus (mg/L) lot 2	47.59 ± 0.19	3170.51 ± 412
Snail mucus (mg/L) lot 3	52.82 ± 0.60	2979.23 ± 443
Helix ir (mg/g)	1.80 ± 0.01	29.75 ± 0.73
Elicina (mg/g)	0.40 ± 0.01	23.95 ± 2.18
Labcconte (mg/g)	1.25 ± 0.01	28.05 ± 2.18

every sample, show the method's exceptional intra- and inter-day accuracy and the low %RSD values, the very good intra- and inter-day repeatability.

3.2.3. Stability

The stability of the studied compounds (allantoin and glycolic acid) in snail mucus and cosmetic creams was studied with spiked samples. Samples were analyzed (three repetitions at each concentration level) at different time intervals by applying the following conditions: (a) after a week in room temperature, (b) after 3 freeze-thaw cycles (freeze at −20 °C – thaw at room temperature), (c) after a week in the refrigerator (4–8 °C) and d) after 6 months at −20 °C. These results are presented in Supplementary Information (Tables S2–S5). Precision (%RSD < 4.16) and accuracy (RE < 4.34%) values show the great stability of the method and samples in tested conditions.

3.3. Quantification of allantoin and glycolic acid in snail secretions and cosmetic creams

To quantify allantoin and glycolic acid in different lots of snail mucus, samples (at a concentration of 50 μL mucus/mL buffer solution) were prepared and were analyzed using the developed method. The method was also applied to the determination of allantoin and glycolic acid in commercially available cosmetic creams, in which mucus is the main ingredient. Analysis of three different lots of mucus and of the cosmetic creams showed that the mucus content in glycolic acid and allantoin is variable (approximately 20% and 10%, respectively between the larger and the lower values). The levels of allantoin and glycolic acid in the cosmetic creams are presented in Table 3; allantoin levels are more variable (40%)

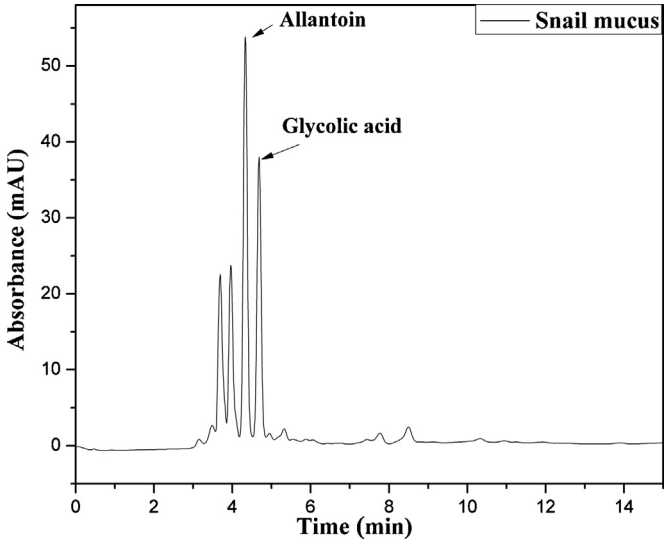

Fig. 1. Separation of allantoin and glycolic acid in snail mucus at concentration 50 μL mucus/mL after dilution in buffer, heating at 60 °C, adjusting the pH to 2.9 and extraction with hexane.

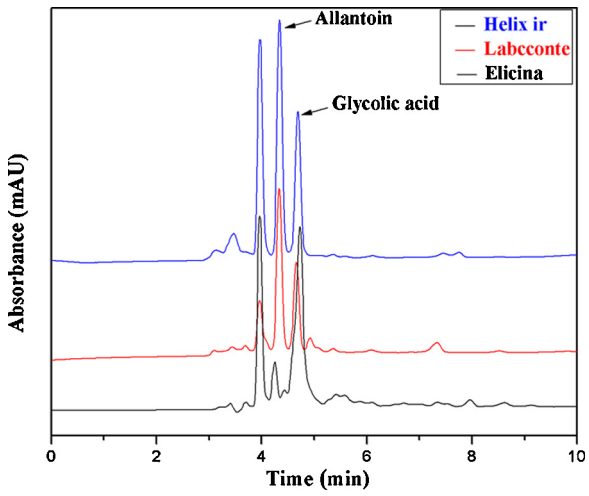

Fig. 2. Separation of allantoin and glycolic acid in three cosmetic creams at concentration 200 mg cream/50 mL after dilution in buffer, heating at 60 °C, adjusting the pH to 2.9 and extraction with hexane.

than those of the glycolic acid (20%). These results are presented in Figs. 1 and 2 as well as in Table 3.

3.4. Comparison to literature methods for analysis of cosmetics

This is the first report of simultaneous determination of allantoin and glycolic acid in mucus and cosmetic creams. All other methods (see Table S6 in Supplementary Information) determine each compound separately.

Analysis of cosmetic creams with our methodology led to the determination of allantoin with a higher precision and sensitivity than other methods [19,20]. Our method concerning glycolic acid determination is not as sensitive as that of Scalia et al. [32], which however used sample pretreatment with strong-anion-exchange columns. In comparison to the ion-pair method HPLC method by Chang and Chang [30] which also did not use exquisite pretreatment, our LLOQ is lower.

4. Concluding remarks

A fast method for simultaneous separation and quantification of allantoin and glycolic acid in cosmetic creams and snail mucus was developed for the first time. Validation of the method showed high recovery, precision, and sensitivity. It allows the rapid, specific and reproducible simultaneous determination of those compounds in complex mixtures such as snail mucus and cosmetic creams, and thus could be of use in the quality control in cosmetic industries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.10.086.

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